Use of the Detection of Anaphase/Telophase Abnormalities to Study Genotoxicity of Solid Particles

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We studied the induction of chromosome missegregation by solid particles during anaphase and telophase. We treated rat pleural mesothelial cells cultured in slide flask during 48 hours with four different glass microbeads (SER 3 % Fe_2O_3, C_3 0.22 % Fe_2O_3, Basalt GH 12.7 % Fe_2O_3 and Basalte GH 8 % Fe_2O_3), anatase (titanium oxide, an isomorphic particle) and crocidolite UICC (an asbestos fibre) during 48 hours. After fixation with a solution of methanol and acetic acid (3:1) and coloration with 3 % of Giemsa, 150 anaphases and telophases were scored and classified into lagging chromatin, bridge and non polar segregation. The results showed that crocidolite at the dose of 7 $\mu g/cm^2$ was a strong inducer of anaphase/telophase abnormalities. The majority of the abnormal segregation was lagging chromatin. Moreover, the four glass microbeads and anatase had no effect at doses up to 20 $\mu g/cm^2$ on chromosome segregation. These results suggest that fibre shape is an important parameter for the induction of chromosome missegregation, whereas there was no significant effect with isomorphic particles with different iron content. The detection of chromosome missegregation showed to be a sensitive test and could be used complementary to other genotoxicity tests.

In Vitro Effects of Glass Microbeads with Different Iron Content on Cultured Rat Pleural Mesothelial Cells

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Different results from the literature have suggested that iron may play a role in the toxicity of some mineral dusts. In our study we tried to determine whether glass microbeads with different iron content exerted any cytotoxic and genotoxic effect on cultured rat pleural mesothelial cells (RPMC). Three samples were studied with different iron 0.02, 3 and 8 %. Cytotoxicity was detected by the measurement of metabolic pathways involving succinate dehydrogenase. Microbeads were internalized by RPMC and produced a reduction of cell metabolism but no cell death. The cytotoxic assay showed that there was no difference in cytotoxicity between the samples. Genotoxicity assays indicated no unscheduled DNA synthesis (UDS) enhancement nor chromosomal aberrations with the microbeads samples indicating that these particles did not produce DNA damage in our experimental conditions. However, a small effect on aneuploidy was detected with microbeads containing the highest iron content. Anatase was also used as control and showed no effect in any assay.

These results demonstrate that three samples of glass microbeads with different iron content are similarly cytotoxic for RPMC, but only one sample of the highest iron content showed a slightly questionable genotoxic effect in aneuploidy. Further investigations are now in progress to confirm these results with microbeads with a higher iron content and to obtain quantitative data on the amount of particles internalized.

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A 20

Mechanism Studies of Silica Toxicity and Carcinogenesis at the Tissue, Cellular and Molecular Levels

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The mechanisms of action of crystalline silica and other particulate minerals are investigated in relation to cell toxicity, fibrogenesis and carcinogenesis, by studies on DNA damage, possible mutagenesis, chromosomal damage, neoplastic transformation, epithelial cell proliferation, carcinogenesis, and altered gene expression. The mineralogic and geochemical characteristics of particulate materials need to be defined in relation to their biological effects, through collaboration of experts in geochemical and biological sciences.

Studies in our laboratory, reported in 4 separate posters, provide evidence for some mechanisms of silica effects and suggest others. Two main in vivo effects of silica are (a) proliferation of fibroblasts leading to progressive fibrosis, and (b) proliferation of alveolar epithelia leading to fibrosis-associated carcinomas. Species differences indicate that these two pathways are controlled by different host factors. In order to define the relationship between fibrogenesis and carcinogenesis induced by silica, we are investigating the role of cytokines. The observed localization of TGF-ß1 precursor in the hyperplastic alveolar type II cells, near silicotic granulomas, suggests that these epithelial cells may in turn stimulate fibrogenesis. Other mediators released by silicotic granulomas, including IL-1, TNF-α, mast cell products and oxygen radicals, require further studies. Silica was internalized by cultured alveolar type II cells and by BALB/3T3 cells. By electron microscopy, most particles were found in the cytoplasm, and a few small particles in nuclei. The significance of nuclear localization remains to be investigated. After internalization, silica appeared no longer cytotoxic to dust-laden cell monolayers, but these cells remained susceptible to lysis induced by new exposure to silica. In BALB/3T3 cells, different silica samples induced neoplastic transformation with a constant pattern, independently of cytotoxicity, suggesting separate mechanisms for these effects. Transformation methods are being developed for rat alveolar type II cells. In vitro studies showed direct DNA damage by silica, mediated by the formation of reactive oxygen. Different silica-induced effects, including damage to cell membranes and organelles, damage to DNA and to chromosomes, cell killing, cell proliferation and neoplastic transformation, may be due to different silica properties and mechanisms. We are investigating the biological effects of silica in relation to surface area, surface charge, presence of iron impurities, and combined exposure with other minerals. The combination of (a) direct genetic damage in the target lung epithelia in vivo and (b) the chronic stimulation of alveolar epithelial cells by mediators produced during fibrogenesis (with possible feed-back from epithelial to mesenchymal cells), may account for silica-induced carcinogenesis in susceptible hosts.

A 21

Flow Cytometric Quantitation of Clearance and Macrophage Burden of Inhaled Polystyrene Beads in Rodents

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The aim of the study was to develop a method to assess the functional ability of the pulmonary clearance system, avoiding the use of radiolabeled materials.

Male Sprague-Dawley rats were exposed by nose-only inhalation to fluorescent polystyrene latex beads (diameter, 1.85 µm), at a concentration of 2 - 15 mg/m^3 for ¼ hour (low burden) or 2½ hours (high burden). Groups of animals were killed immediately after exposure and at subsequent time-points to examine the rate of removal of inhaled beads. Respiratory system organs were removed and placed in a basic solution to digest the tissue. The beads, which are not destroyed by the tissue digest solution, were counted on a flow cytometer. The flow cytometer was also used to analyze lavage fluid from exposed animals.

The exposures resulted in initial lung burdens of approximately 3.5 and 20 million beads for low and high burdens, respectively. The rate of removal of the beads from the lungs showed half-lives of around 55 days. There were dose-related proportions of lavaged macrophages (Mϕ) containing beads: at 24 hours after exposure this figure was 5% for the low burden and 25% for the high burden. At 48 hours after the high burden exposure, 55% of the Mϕ that contained beads had 1 bead, 21% 2 beads, 10% 3 beads, 4% 5 beads, and 5% 6 or more beads.

The use of these inert particles to assess lung clearance provides advantages in ease of use, lack of potential contamination, and a novel quantitative ability when compared to other methods. The lavage techniques provide a novel method for *in vivo* assessments of phagocytosis.

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A 22

A Five Year Follow-Up of TNF-Release and Serum Type III Procollagen as Biological Markers for Pneumoconiosis

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Although many theories to explain the fibrogenesis due to mineral dust exposure have been elaborated, it is not clear why certain coal workers develop pneumoconiosis and why others, equally exposed, do not. During the past decade two major new insights were put forward by the elucidation of (i) the role of free radicals and (ii) the crucial role of alveolar macrophage (AM)-derived cytokines such as TNF-α. The significance of these mechanisms as markers for individual susceptibility was investigated by our laboratory in several cross-sectional studies among coal miners (review: Borm *et al.*, Exp Lung Res 16:57, 1990). These studies suggested that measurement of anti-oxidant enzymes (AOE) in red blood cells and serum type III procollagen peptide (PIIIP) might be more sensitive diagnostic parameters than routine chest X-rays (Janssen *et al.*, Exp Lung Res 18:1, 1992). Moreover, we suggested that the *ex-vivo* release of TNF-α might be a useful parameter to discriminate individuals susceptible to CWP. A follow-up study was started in October '92 to test our former hypotheses. All (156) miners previously investigated will be screened for chest X-ray, lung function, exposure history and the biological markers TNF-α, PIIIP and AOE. Data analysis (February '93) will concentrate on the relation between prospective individual changes in standardized X-ray and biological markers. Within its statistical limits this molecular epidemiological study will generate definite conclusions about the diagnostic as well as prognostic power of these markers in CWP or pneumoconiosis in general.

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A 23

One Year Exposure to Diesel Engine Exhaust Causes Lung Tumors

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To know whether one year exposure to diesel exhaust is enough to cause lung tumors if exposure begins early in life, we planned a long-term inhalation experiment.

Fischer 344 SPF female rats were used, and were divided into 4 groups. Group 1 (G1) was exposed at 4 weeks for 6 months. In group 2 (G2) exposure began at 4 weeks for 1 year. In group 3 (G3) exposure began at 4 weeks and continued for 2 years. Group 4 (G4) was the unexposed control group. Finally, the animals were examined 2.5 years after the beginning of the treatment.

Diesel engine exhaust was generated at a steady state condition (1000 rpm, and 90 % load). Concentrations of particles were 4.73 + 0.74 mg/m^3 by dilution and introduced into the animal chambers. Exposure conditions for the rats were 15 hours/day, 3 times/week. Animals which died after 18 to 30 months were included in the evaluation. In the control group (G4), 5 rats of 48 had 6 pulmonary adenomas, in G1 1 of 45 had 1 cancer, in G2 8 of 42 rats had 9 lung tumors (7 adenomas and 2 cancers), and in G3 6 of 49 rats had 8 lung tumors (7 adenomas and 1 cancer). Incidence of lung tumors in G2 is statistically higher than that in G1 (p 0.05). The degree of anthracotic changes and inflammatory changes such as fibrosis is more severe in G3 compared with G1 or G2. Incidence of tumors other than lung tumors was not different among the groups. We suspect that if exposure to diesel engine exhaust begins early in life (4 weeks after birth), 1 year is enough to cause lung tumors. It is also suspected that the developing lung is susceptible to toxic substances.

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Subchronic Inhalation Exposure of Rats to Carbon Black and/or Irritant Gases

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Three groups of female Wistar rats were exposed 18 hours/day, 5 days/week for two months either to 6 mg/m^3 carbon black (mass median aerodynamic diameter 1.1 µm, BET surface area 230 m^2/g, extractable organic mass 0.04 %), to a mixture of irritant gases (5 ppm SO_2, 5 ppm NO_2, 3 ppm formaldehyde) or to carbon black (CB) and irritant gases (IG) combined.

The following investigations were performed at the end of exposure, to assess toxic effects in the respiratory tract: measurement of the lung wet weight and the particle mass retained in the lungs; biochemical and cytological assays of broncho-alveolar lavage fluid (BALF); mechanical lung function measurements using the whole-body plethysmograph and the anesthesized spontaneously breathing rat.

At the end of exposure, the lungs of the CB-exposed rats contained almost 3 mg particles/lung but mechanical lung functions (e.g. compliance) did not show significant impairment. The exposure to IG with and without CB led to some changes in the flow-volume curve.

Besides the lung weight, also the number of macrophages, granulocytes, lymphocytes and the concentration of some cell enzymes (lactate dehydrogenase, ß-glucuronidase) as well as total protein and hydroxyproline in BALF were significantly increased in CB-exposed animals. None of these parameters showed a treatment-related alteration after IG inhalation.

Comparing the effect of CB alone to the mixture of CB and IG, the latter exposure showed less dramatic alteration in the respiratory tract. This result may be explained by the 25 % lower retention of CB in the lungs of rats inhaling CB together with IG. A reduced breathing frequency caused by the irritating effect of the inhaled gas mixture might be responsible for the lower particle deposition. Chronic exposure to coal tar/pitch aerosol plus IG resulted in a significantly lower lung tumor rate compared to the exposure to coal tar/pitch aerosol alone (Heinrich et al., 1992).

Long-Term Inhalation Exposure of Rats and Mice to Diesel Exhaust (DE), Carbon Black (CB), and Titanium Dioxide (TiO_2)

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Female Wistar rats were exposed 18 hours/day, 5 days/week for 24 months to either DE containing 7.5 mg/m^3 diesel soot (extractables 40 %, BaP 3.9 µg/g, 1-NP 19 µg/g, MMAD 0.25 µm, surface area 18 m^2/g, after extraction 96 m^2/g) or 7.5 mg/m^3 CB or 7.5 mg/m^3 TiO_2 (Anatase, primary particle size 15 nm, MMAD 0.8 µm specific surface 48 m^2/g); after the two year exposure period the animals were kept for another 6 months in clean air. The exposure concentration of CB and TiO_2 were increased during the course of the experiment (final concentration 12 and 10 mg/m^3, respectively) to try to meet the particle lung load of the DE-exposed rats. Satellite groups were used to determine the amount of particle mass retained in the lungs of the various exposure groups at different time points during the exposure period. Two other groups of rats were exposed to DE containing 2.5 and 0.8 mg/m^3, respectively. Also, NMRI mice inhaled the above mentioned DE, CB, and TiO_2 concentrations. Furthermore, NMRI and C57Bl mice were exposed to DE containing 4.5 mg/m^3 diesel soot and DE without soot particles.

Some major results: Lung tumors occurred in rats after exposure to DE, CB and TiO_2 and the tumor rate increased with increasing normalized exposure concentrations independent of the kind of particle inhaled. No lung tumors were found after exposure to 0.8 mg/m^3 diesel soot but, one lung tumor-bearing rat occurred in the controls. Alveolar lung clearance was significantly impaired already after 3 months of exposure to 0.8 mg/m^3 diesel soot; at this time the lung contained 0.6 mg diesel soot. This amount increased to 6 mg after 24 months whereas the exposure to 7.5 mg/m^3 diesel soot led to a lung particle load of 60 mg. Despite higher exposure concentrations of CB and TiO_2 compared to diesel soot, CB and TiO_2-exposed lungs contained about 20 mg less particles than the DE exposed lungs after an experimental time of 24 months. None of the various groups of exposed mice showed a significantly increased lung tumor rate. The experimental time for the NMRI mice was 23 months (30 % lung tumors in controls) and for the C57Bl mice 30 months (5 % lung tumors in controls), at the most. The mortality rate, the particle load/g lung and the load increase over time was quite similar in C57Bl mice exposed to 4.5 mg/m^3 diesel soot and in rats exposed to 7.5 mg/m^3 diesel soot.

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In Vitro and In Vivo Toxicity of Coal Fly-Ash and Lytag Dust

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A product of coal-fired power stations is fly-ash which can be processed to several secondary products. One example is Lytag, a light weight aggregate fly-ash from electrostatical precipitators (EPS), sintered to grains and mostly used for construction purposes.

Fly-ash consists mainly of clay minerals and can contain various potentially (geno)toxic or carcinogenic compounds such as heavy metals, arsenic, α-quartz, and radioactive elements in low concentrations. Occupational exposure to coal fly-ash necessitates understanding of the potential adverse health effects.

In the present study, various fly-ash samples were evaluated in vitro with respect to toxicity on bovine macrophages (trypan blue exclusion) and human erythrocytes (haemolysis). The results showed that the in vitro toxicity was strongly correlated to the amount of α-quartz present in the samples. The in vitro results were verified in vivo using the bronchoalveolar lavage fluid analysis assay in male Wistar rats. Groups of 16 rats were instilled via the trachea with either saline, 10 or 50 mg/kg α-quartz (positive control), 10 or 50 mg/kg TiO_2 (innocuous dust) or either 2, 10, or 50 mg/kg fly-ash from low NO_x burners or Lytag dust. At days 7, 14, and 28, 4 rats of each group were sacrificed, lungs were lavaged, and the lavage fluids were analysed for selected biochemical and cellular markers of toxicity. In addition, the lungs of 4 rats per group were examined histopathologically. The results of the present study showed that (a) α-quartz treatment induced dose-related progressive lung toxicity, (b) 50 mg/kg TiO_2 induced pulmonary responses related to overload mechanisms and (c) fly-ash and Lytag showed effects comparable with those of TiO_2, except for increased lymphocyte numbers in lavage fluid upon treatment with either substance. A more detailed presentation and discussion of the results will be given during the meeting.

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20573581123

Transformation of the Fetal Rat Alveolar Type II Cell Line, FRLE, by a Mutated K-*ras* Gene, and Studies on Silica Uptake and Toxicity in FRLE Cells

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Alveolar carcinomas were the most common type of fibrosis-associated lung cancer induced by silica in our rat model. In order to study mechanisms of silica-induced carcinogenesis in alveolar type II cells, we sought an appropriate cellular model. The recently isolated fetal rat epithelial cell line, FRLE (Leheup et al., 1989), that retains the characteristics of alveolar type II epithelia, was selected for these studies. Characterization of this cell line in our laboratory indicated that it was indeed a suitable model. Electron microscopic studies of silica uptake and effects on FRLE cells showed, at the ultrastructural level, that silica particles were internalized and localized in the cytoplasm, mostly in membrane-bound vacuoles (phagolysosomes). A few small particles were also localized inside the nucleus. Damage to cytoplasmic organelles, resulting from the internalized silica particles, was observed.

The FRLE line, at passage 38, was found to be aneuploid with most chromosome counts in the hypotetraploid range, but it was not tumorigenic in nude mice. No assay is presently available for the induction by carcinogens of neoplastic transformation in rat alveolar type II cells.

In order to obtain a neoplastic phenotype of this cell type, we transformed FRLE cells by transfection of a K-*ras* oncogene carrying a codon 12 mutation. This K-*ras* mutation has been found frequently associated with human lung adenocarcinomas, and therefore was considered to be appropriate for transformation of rat alveolar epithelial cells. We propose to use the *ras*-transformed lines as reference standards, in order to develop criteria for the selection of transformed colonies and to develop a transformation assay suitable for testing silica and other minerals.

FRLE cells were lipofected with plasmid pZip•K12•Cys, containing a K12 Gly→Cys mutated c-K-*ras* sequence inserted into the pZip•Neo shuttle vector (kindly provided by Dr. C. C. Harris, Lab. of Human Carcinogenesis, National Cancer Institute, NIH). After 4 weeks in Geneticin-containing selective medium, 7 colonies from the pZip•Neo controls and 18 colonies from the pZip•K12•Cys treated cells were isolated individually, expanded into cell lines and cryopreserved. In 14 out of 18 *ras*-transfected lines, the colony morphology was found to be aberrant. Five of these lines have been so far injected s.c. in nude mice, and were all rapidly tumorigenic, whereas control colonies were not. Expression of the *ras* gene, growth properties and tumor pathology are being studied in relation to colony morphology and ultrastructure. Development of a transfomation assay in FRLE cells, suitable for silica and other carcinogens, is in progress.

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Effects of Different Silica Samples in BALB/3T3/A31-1-1 Cells: Uptake, Cytotoxicity and Neoplastic Transformation

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The objectives of this study were: (1) to evaluate the ability of crystalline silica samples to induce neoplastic transformation in quantitative assays, using the mouse embryo cell line BALB/3T3/A31-1-1 (passage 12); (2) to investigate mechanisms responsible for silica uptake, cell toxicity, transformation, tumorigenicity, chromosomal changes, and altered gene expression. The silica samples included: Min-U-Sil 5 quartz (MQZ), hydrofluoric-acid-etched MQZ (HMQZ); DQ12; F600; Chinese standard quartz (CSQZ); cristobalite; tridymite. Non-silica dusts included ferric oxide, anatase and rutile. The cells exposed to dust showed phagocytosis of numerous dust particles. Electron microscopic studies showed location of MQZ particles in the cytoplasm, mostly in membrane-bound vacuoles (phagolysosomes). Damage to organelles was evident and myelin bodies were present. A few small silica particles were observed inside nuclei. Tests for cytotoxicity (measured by reduced colony-forming efficiency) showed a trend that correlated cytotoxicity with surface area and surface charge, but with exceptions, notably the low toxicity of F600. In tests with MQZ, when dust-laden cells reached confluence, no further toxicity was apparent, but exposure of such cell monolayers to newly added MQZ determined extensive cell lysis.

All 5 tested quartz samples induced significant levels of neoplastic transformation, showing dose-dependent increases in the frequency of morphologically transformed foci at lower tested doses and a plateau at higher doses. The plateau levels reached by the 5 tested samples did not differ substantially (maximum transformation frequencies per 10^5 cells ranging from 53.2 for MQZ to 28.3 for HFMQZ). F600 induced a transformation frequency of 41.2 at 50 $\mu g/cm^2$, even though it had minimal cytotoxicity. The present results are consistent with a range of transformation responses common to all tested quartz samples. Cells from all tested transformed foci, injected s.c. in nude mice, grew as sarcomas. Using cytogenetic analysis, all silica-transformed cell lines showed 1 to 5 additional marker chromosomes each, of types not seen in untreated control lines. The frequencies of transformed foci were low in untreated controls (0.13 foci per 10^5 surviving cells). Increased expression of one or more of 5 genes (*p53*, *myc*, H-*ras*, K-*ras*, and *abl*) was observed in 5 out of 6 tested cell lines transformed by MQZ, and in 3/3 lines transformed by HFMQZ, but in 0/3 lines transformed by F600. No transforming activity was found for ferric oxide and anatase (both non-toxic) and for rutile (more toxic than MQZ). Combined exposure (1:1 w/w) of each of these dusts with MQZ showed that the non-toxic dusts inhibited MQZ toxicity, whereas rutile markedly enhanced MQZ toxicity. Transformation assays are in progress.

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A 29

DNA Damage In Vitro Induced by Crystalline Silica Dust Samples Characterized for Surface Area and Surface Charge

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The ability of silica to cause direct DNA damage in vitro was investigated. Quartz samples included: Min-U-Sil 5 (MQZ), HF-etched MQZ, DQ12, F600, Chinese standard quartz (CSQZ), and a series of Min-U-Sil particle size fractions. In addition, cristobalite and tridymite were tested. A simple electrophoretic DNA double strand breakage assay was developed. Aqueous suspensions of silica were added to solutions containing 10 µg Hind III digested λ DNA. Aliquots of 1 µg DNA were removed at selected times. Following electrophoresis in agarose, the gels were stained with ethidium bromide and photographed. Smearing of the discretely sized bands indicated ongoing DNA damage. Modifiers of free radicals, such as superoxide dismutase, hydrogen peroxide (H_2O_2), metal chelators and free radical scavengers, were added to reaction mixtures to test their effects on the rate of DNA damage. All buffers were previously chelexed to remove trace iron. Oxygen consumption by silica in aqueous suspension was detected using an oxygen electrode. The effect of anoxic conditions on in vitro DNA damage was determined using a specially designed sealed reaction vessel purged with argon. Silica caused detectable changes in DNA band sizes after 20 days of incubation in 10 mM phosphate buffer at 37°C, whereas DNA was stable in buffer alone. DNA damage was accelerated by superoxide dismutase and greatly accelerated by H_2O_2. Coincubation with iron chelators, iron transport proteins and hydroxyl radical scavengers modified the resulting DNA damage. Silica-derived superoxide free radicals, produced in aqueous solution, may generate hydroxyl radicals after dismutation into H_2O_2 and conversion via catalysis by iron surface impurities on silica.

Results of a sensitive assay for the formation of thymine glycol (an oxidized DNA base) generally corresponded with those of the DNA strand breakage assay (in collaboration with S.P. Markey, NIMH).

Fourier transform infrared spectroscopy was used to obtain spectra for aqueous solutions of silica, calf thymus DNA and the combination of silica and DNA. Computer generated subtraction images for individual components from the spectrum of DNA and silica together were analyzed for shifts in the spectrum and revealed that silica binds to the phosphate backbone of the DNA strand.

A method was developed for measuring anionic charges on silica surfaces by the binding of the cationic dye Janus Green B, which was found to be directly proportional to the surface area of tested silica samples. The level of DNA damage, reported above, was roughly inversely proportional to surface area and surface charge.

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A 30

Immunohistochemical Localization of TGF-ß1 in Rat Lung Silicosis, Adjacent Alveolar Type II Hyperplasia and Tumors

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Immunohistochemical localization of TGF-ß1 was studies in the lungs of F344 rats given crystalline silica (12 mg Min-U-Sil 5 quartz) by single intratracheal instillation. This treatment induced granulomas with progressive fibrosis, adjacent alveolar type II hyperplasia and alveolar cell tumors (a total of 8 adenomas and 88 carcinomas was found in 96 rats examined between 11 and 26 months). Rabbit polyclonal antibodies to synthetic peptides corresponding to the first 30 amino acids of mature TGF-ß1, anti-LC(1-30) and anti-CC(1-30), were used, respectively for localization of intracellular and extracellular TGF-ß1. An antibody to a peptide corresponding to amino acids 266-278 of the TGF-ß1 precursor sequence, anti-Pre(266-278), was used to detect the TGF-ß precursor with the latency associated peptide (LAP). Immunoreactivity to intracellular TGF-ß1 was localized in fibroblasts and macrophages at the periphery of silicotic granulomas and in the stroma adjacent to hyperplastic type II cells, especially at sites of collagen deposition. Immunoreactivity to extracellular TGF-ß1 was localized in the connective tissue matrix of the granulomas and of the stroma of both hyperplastic type II cells and adenomas and, occasionally, in the fibrous stroma of adenocarcinomas. Immunoreactivity to anti-Pre showed a consistent punctate pattern of intracellular localization, in hyperplastic alveolar type II cells and their proliferative lesions adjacent to granulomas, in adenomas, but not in well differentiated adenocarcinomas. Poorly differentiated carcinomas showed occasionally, at the periphery, a weak reactivity to anti-Pre.

Ferric oxide treated rat lungs, used as controls, showed no progressive granulomatous reaction, no epithelial hyperplasia and no lung tumors; they showed no demonstrable reactivity to TGF-ß1. Control immunostaining with normal rabbit serum IgG was always negative.

The localization of mature TGF-ß1 in fibroblasts at the periphery of granulomas, near areas of hyperplastic alveolar type II cells positive for TGF-ß precursor, suggests a functional relationship between these two cell types. TGF-ß is known to stimulate fibroblasts to synthesize collagen. The hyperplastic type II cells appear to be the sites of production and secretion of TGF-ß1, which may regulate their own growth and differentiation and also mediate the production of extracellular TGF-ß1 associated matrix. The lack of reactivity to TGF-ß1 precursor in the adenocarcinomas is consistent with loss of normal cellular differentiation and function. TGF-ß1 appears to have a pathogenetic role in silica induced mesenchymal and epithelial lesions. The role of TGF-ß and other cytokines in silica induced carcinogenesis requires further investigation.

2057358125

A 31

Response of the Rat Lung to Inhaled Versus Instilled Particles

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The potential pulmonary toxicity of poorly soluble airborne dusts generated in industrial and environmental settings is often evaluated by inhalation studies in rodents. Studies using intratracheal instillation of particles have been suggested as a quicker and less expensive alternative. We have conducted a study to compare the inflammatory response of the lung to instilled versus inhaled particles. In one study, female F344/N rats, 11-13 wk of age, were exposed for 6 hr/day, 5 days/wk for 4 wk by inhalation to 0, 0.1, 1.0, or 10 mg/m^3 of either α-quartz (toxic particle) or TiO_2 (relatively low toxicity) and the lung burdens determined at one week after the end of the exposure. The lungs were evaluated by analysis of bronchoalveolar lavage fluid (BALF) at 1, 8, and 24 wk after the end of the exposure and by histopathology at 24 wk. In a second study, rats were exposed by instillation to the lung burdens present in the rats at one week after the inhalation exposure and the rats were evaluated in the same manner as in the inhalation study. In general, the acute inflammatory response was similar by either method of exposure. With lung burdens up to 0.75 µg/g lung, the TiO_2 elicited no changes in BALF parameters at any time by either method of exposure. The BALF changes elicited by α-quartz were of approximately the same magnitude by either exposure method and granulomatous lesions occurred in both sets of animals. One difference observed was that the instillation exposure tended to elicit the influx of higher numbers of neutrophils than did the inhalation exposures. The study indicates that the use of intratracheal instillation is an adequate method of exposure in short-term studies to compare the relative pulmonary toxicity of a series of particles or industrial preparations. (Research supported by U.S. DOE/OHER and P & G Co. under FIA DE-FI04-87AL44004.)

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Cyto- and Genotoxic Effects of Insoluble Particles In Vitro

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Inhaled solid particulate materials have been shown to cause airway tumors in the rat. The possibility that this carcinogenic potency may depend on the particles' own physical or chemical properties rather than on particle-bound organic chemicals (e.g. in the case of diesel soot) has recently become a controversial issue. Chemically inert particles have therefore been investigated for their biological effects on an in vitro system of hamster airway epithelial cells, which represent a target cell model for airway toxicity and carcinogenesis.

Carbon black and titanium dioxide particles were compared with respect to their ability to induce cellular alterations such as cytotoxicity, anchorage-independent cell transformation, chromosomal lesions or cytoskeletal disturbances. Both particles types led to distinct toxic effects at high concentration levels. Cytoskeletal components such as the vimentin and the actin skeleton were influenced by both types of particles, whereas the tubulin network remained unchanged. Chromosomal damages were monitored by evaluating induction of micronuclei. Both particulate substances were shown to induce enhanced micronucleus frequencies as compared with the untreated control, although the concentrations used to induce micronuclei were in the very low non-toxic range, as was shown by evaluation of the mitotic indices in treated cell populations. The majority of micronuclei induced contained no kinetochor and were thus suggested to originate from clastogenic activity of the particles examined. Both particulate specimens also induced anchorage-independent cell transformation of the hamster lung cells.

The study shows that chemically inert solid particles exhibit strong biological activities in vitro. On the cellular as well as the subcellular level, even very low concentrations of the dusts induced severe changes which might have implications for the carcinogenic potency of particulate dusts.

A 33

Chronic Inhalation Toxicity of Talc in F344/N Rats and B6C3F$_1$ Mice

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F344/N rats and B6C3F$_1$ mice were exposed to aerosols of non-asbestiform, cosmetic-grade talc (mass median aerodynamic diameter ~ 3 µm) 6 h/day, 5 days/wk for 103 or 104 wk for mice, 113 wk for male rats, and 122 wk for female rats. The exposure concentrations were 0, 6, and 18 mg talc/m^3. The lung burden accumulated with time and reached levels of about 20 - 30 mg talc/g of control lung in rats and mice exposed to 18 mg/m^3, and about 10 mg (rats) and 3 mg (mice)/g of control lung in animals exposed to 6 mg/m^3. Body weights were slightly depressed in rats exposed to 18 mg/m^3, but unaffected in other groups of rats and mice. Survival was not affected by the exposure. Lung weights were elevated in both rats and mice exposed to 18 mg/m^3. Pulmonary function measurements showed a dose-related progression of changes consistent with restrictive lung disease. Histopathological examination of the lungs of rats showed progressive dose-related, non-neoplastic changes in the lung consisting of granulomatous inflammation, peribronchial histiocytic hyperplasia, alveolar epithelial hyperplasia, squamous epithelial metaplasia, focal interstitial fibrosis, and squamous cysts. In mice, the non-neoplastic lung lesions were also dose-related and consisted of chronic inflammation and macrophage hyperplasia. The incidence of lung tumors (both bronchiolar-alveolar adenomas and carcinomas) was significantly increased only in female rats exposed to 18 mg/m^3. Both male and female rats exposed to 18 mg/m^3 had increases in pheochromocytoma (combined benign and malignant) of the adrenal gland. There were no significant increases in neoplasms in mice. (Research supported by the National Toxicology Program of NIEHS via Interagency Agreement Y01-ES-20088 with the U.S. DOE/OHER under Contract No. DE-AC04-76EV01013.)

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A 34

Effect of Arsenic Exposure on Alveolar Macrophage Function

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Alteration in function of pulmonary alveolar macrophages (PAM) was evaluated following *in vivo* and *in vitro* exposure to slightly soluble forms of arsenic. Male Sprague-Dawley rats were used throughout. One day following intratracheal instillation of 1 mg/ml (as arsenic) of either arsenic trisulfide (As(III)) or calcium arsenate (As(V)), PAM were lavaged and analyzed for alterations in prostaglandin E_2 (PGE_2), and tumor necrosis factor (TNF-α) production. Bronchoalveolar lavage fluid (BALF) was also analyzed for presence of PGE_2 and TNF α. In addition, PAM from control animals were exposed, *in vitro*, for up to 24 hrs to concentrations of 1 µM to 4mM arsenic. No differences in cell number or in differential cell count were seen in the arsenic groups as compared to controls. In addition, there were no differences in BALF PGE_2 or TNF-α. PAM lavaged from animals exposed to arsenic *in vivo* showed no alteration in basal or lipopolysaccharide (LPS)-induced production of PGE_2. However, PAM lavaged from arsenic exposed animals had decreased basal release of TNF-α. *In vitro*, arsenic exposure had no effect on PGE_2 production. However, both arsenic species caused suppression of LPS-induced release of TNF-α. Inhibition of TNF-α occurred at lower concentrations of As(III), 50 % inhibition at 20 µM, compared to As(V), 50 % inhibition at 125 µM. Differences between As(III) and As(V) indicate that different mechanisms and/or potencies exist between the two arsenic species. Reduction of *in vivo* and *in vitro* production of TNF-α by arsenic may indicate an ability of arsenic to compromise host defense against infections. (Supported by ES 05561)

20573581279

A 35

Assessment of Maximum Exposure to Soil Contaminants During Gardening

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To evaluate the additional exposure to soil contaminants during gardening dust measurements were carried out in a housing area in Hamburg, where the soil was highly contaminated with arsenic, heavy metals, and dioxins. Worst case conditions were simulated by one or two persons sweeping and raking an ungrown area for a total of nearly seven hours while the weather was dry and sunny with a temperature of 25° C. Imitating the conditions of a child, which is playing while somebody is working in the garden nearby, a personal air sampler (membrane filter, pore size 0,8 µm, flow velocity at the filter 1,25 m/s.) was installed at the leg of the gardening person. A small filter instrument was installed within the working area (quartz fiber filter, flow velocity at the filter 0,8 m/s) collecting total suspended particles (TSP) at 1,5 m. To evaluate the background level of dust concentration during sampling time a second small filter instrument and an additional personal air sampler were operated at a reference location nearby.

The dust concentration caused by whirled up soil particles during gardening was 5,7 mg/m^3 (personal air sampler). The amount for arsenic and lead in this sample was enriched by a factor of 2,5 compared to soil analyses (As: 83 mg/kg soil; Pb: 259 mg/kg soil) of the same place. The dioxin concentration in the air was estimated assuming a soil concentration of 1000 ng I-TEq/kg and considering the same enrichment factor as found for lead and arsenic.

Assuming that a person would be working in a garden three hours daily during forty days per year the mean daily intake within a year was calculated and compared to the background levels.

The additional mean daily intake (AMDI) by the dust development through gardening for arsenic was 6,6 ng/kg/d (adult; background level: 1,43 ng/kg/d). For a child it was 11,2 ng/kg/d (background level: 2 ng/kg/d). For lead the AMDI was 24 ng/kg/d (adult; background level 33 ng/kg/d) and 40 ng/kg/d (background: 46 ng/kg/d) for a child, respectively. For dioxins the AMDI was 0,09 pg I-TEq/kg/d (adult; background level: 0,02 pg/kg/d), and for a child 0,14 pg I-TEq/kg/d (background level: 0,03 pg/kg/d).

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A 36

Effects of Single and Combined, Subchronic Exposure to Ozone and Soot Particles on Pulmonary Surfactant Phospholipids, Associated Protein A and on Surface Activity in Rat Lung Lavage Fluid

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It has been shown that the pulmonary surfactant system being important for lung mechanics as well as for non-specific alveolar defense mechanisms is affected by a variety of inhaled pollutants. Only rare information is available about the effects of combined exposure to ambient urban pollutants. Seven-week-old, adult male Wistar rats were subchronically exposed to either ozone (0.5 ppm) or soot particles (0.15 - 1.5 mg) or a combination of both. Soot particles were instilled intratracheally and whole body ozone exposure performed in stainless steel inhalation chambers. At end exposure lung lavage fluid (LLF) was collected, surfactant phospholipid content determined by thin layer chromatography and surfactant-associated protein A (SP-A) assessed by an ELISA. LLF surface tension measurements were performed using the pulsating bubble surfactometer. No significant results were observed in the ozone exposure group except an increase in the minimal surface tension (MST) ($p>0.001$). In animals exposed to soot particles total phospholipid content (TPL) and MST were increased The relative phosphatidylcholine content was decreased ($p<0.05$) whereas the relative content of phosphatidylglycerol and phosphatidylethanolamine proved to be increased ($p<0.05$). In the combined exposure group TPL ($p<0.001$), SP-A content ($p<0.01$) and MST ($p<0.001$) were increased. No changes were detected in terms of mechanical lung function (respiratory rate, - compliance, resistance, flow-volume curve) and gas exchange (CO-diffusion capacity) as assessed by using whole body plethysmography in any of the exposure groups.

From these results we conclude that exposure to soot particles may affect pulmonary surfactant in rats to a greater extent than exposure to ozone at a high ambient concentration.

A 37

Morphometric Evaluation of Inhaled Particles in Sections of the Lung

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Traditionally, pulmonary toxicity has been evaluated by conventional histology. Quantitation, however, offers a number of advantages. It provides a more rigorous comparison of the effects of drugs at different concentrations and demonstrates changes that are individually too small to be detected by qualitative means but collectively may have considerable effects.

Some authors describe methods for stereological evaluation of histological parameters of the lung. All these methods are time-consuming, since many manual interventions are required at each step of the process. In addition the sampling strategy must take into account where changes are likely to occur. Differences might be anticipated between left and right lung, lobes and upper or lower regions. Within the gross anatomical units, changes might be localised within parenchyma, airways or vasculature. Normal parenchyma is considered to have an isotropic organisation, whereas drug-induced changes often have a selective distribution that will necessitate a systemic approach of sampling. For parenchymal morphometry it is important to evaluate their volume density. Because of their orientation the volume density must be determined on samples throughout the whole lung. Accordingly, the whole lung is sectioned systematically for manual evaluation. This means: quantitative morphometric methods are bound up with a large amount of sampling areas.

The development and use of an automatic method - by an image analysing system - for the detection of changes induced in lung compartments by inhaled particles is presented in this paper.

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A 38

Goblet Cell Metaplasia in Rat Lungs after Chronic Exposure to Particles

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After long-term inhalation of Printex 90 and titanium dioxide (7,5 mg/m^3; 6 h/d; 30 mo), goblet cell metaplasias occurred in the peripheral lung tissue of 11/1700 female SPF Wistar rats (strain CRL: (Wi) BR). The majority of goblet cell metaplasias was seen at the bronchiolo-alveolar junction as round, mucus-filled nodules with a diameter of 1 - 20 mm. Their periphery consisted of a single layer of well differentiated columnar goblet cells. In central mucus-filled areas, alveolar epithelium and septae were often markedly reduced. Mucus and goblet cells stained positive with PAS and Alcian blue, both at pH 2.5 and 1. Goblet cell metaplasias have been reported in pulmonary airways of man and hamsters, but have rarely been described in the peripheral lung tissue of rats.

A 39

Phagocytosis and Chemotaxis of Rat Alveolar Macrophages after a Combined or Separate Exposure to Ozone and Carbon Black

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A combined exposure to ozone and diesel soot may induce more severe health effects than an exposure to the single components. In this study, rats were treated by ozone or carbon black (CB) alone as well as in a combination. Male Wistar rats were exposed in the following 6 groups: clean air, 0.5 ppm ozone, 0.15 mg CB, 0.5 mg CB, 0.5 mg CB/0.5 ppm ozone and 1.5 mg CB/0.5 ppm ozone. After the intratracheal administration of CB, animals inhaled ozone for 2 months, 6 h/day, 5 days/week. The effects on the lung were investigated including measurements on lung mechanics, surfactant, histopathology and cellular and soluble parameters of the bronchoalveolar lavagate. From these investigations only the results of two methods will be presented in this paper, i.e. the phagocytosis and the chemotactic migration capability of alveolar macrophages. Phagocytosis was measured by a flow cytophotometer using commercial latex particles (diameter: 0.9 µm) and chemotaxis by the under agarose assay using zymosan-activated autologous serum as chemoattractant. Results: In the phagocytosis assay, the number of phagocytizing macrophages decreased significantly in the CB-exposed groups whereas the ozone group remained at control level. After ozone exposure an increased uptake of particles per macrophage and an increased release of oxygen radicals was observed; the opposite result was observed in the CB groups. Chemotactic migration was only observed at macrophages without phagocytized CB particles; ozone did not impair the migration compared to controls. Conclusion: Ozone stimulates the phagocytic activity of alveolar macrophages, CB impairs phagocytosis as well as chemotactic migration. At these exposure levels no synergistic effect was observed by the methods used.

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A 40

GM-CSF Stimulated Proliferation of Rat Alveolar Macrophages and the Effects of Differing Particulate Burden Levels in Alveolar Macrophages on the Proliferative Response

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Increasing evidence has indicated that even relatively innocuous particles are gradually redistributed among the lung's population of alveolar macrophages (AM) at times well after they were originally deposited in the lung. One mechanism that may underlie this "particle redistribution phenomenon" is the *in situ* replication of particle-containing AM and the allocation of particles from parent AM to progeny cells. Little is known, however, as to how the abilities of AM to proliferate may be affected by the containment of particles; conceivably, AM proliferation may be a particle burden-dependent process. In the current study, we set out: 1) to develop an assay system that provides suitable conditions for rat (Fischer-344) AM to proliferate *in vitro*, and 2) to assess the proliferative abilities of AM that contain differing burdens of phagocytized particles. In the first component of the study, rat AM were found to increase up to ~20 to 30-fold in number over a 7 d period when cultured on a collagenated surface in the presence of rmGranulocyte-Macrophage Colony Stimulating Factor (GM-CSF), with the magnitude of such growth occurring in a GM-CSF concentration-dependent manner. In the second component of the study, rats were intratracheally instilled with 2 x 10^8 fluorescent, polystyrene microspheres (~2 µm dia.), and several days thereafter their lungs were lavaged. The lavaged AM were analyzed using a multiparameter flow cytometer, and the AM were sorted according to their relative burdens of microspheres, i.e., low burdens, medium burdens, high burdens, based on their levels of fluorescence intensities. Control cells consisted of AM that were lavaged from the lungs of untreated animals and also passed through the flow cytometer. The sorted AM were then cultured in the presence of 0.3 ng/ml GM-CSF and cultured up to 15 days thereafter. Control cells and the sorted AM all replicated similarly; no evidence was found to indicate that AM proliferation is either stimulated or compromised in AM as a function of their particulate burdens. In this regard, the results of this study are like those obtained in a recent, similar study in which the effects of differing cellular burdens were examined using the J774.A macrophage cell line (Lehnert et al., *J. Aerosol Med.*, in press). Our overall findings indicate that AM have a marked proliferative capacity when suitably stimulated to enter cell cycle, and they suggest that AM proliferation is not affected by the containment of phagocytized particles that have low intrinsic cytotoxicity. [This work was funded and conducted under the auspices of the U.S. Department of Energy.]

A 41

Influence of Soot-Associated Organic Compounds on Pulmonary Carcinogenicity of Diesel Exhaust in Rats

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Previous studies have shown that inhalation of high concentrations of diesel exhaust (DE) is carcinogenic in rats and the response is linked to DE soot. Furthermore, DE exposure may increase lung DNA adduct levels in rats, and the organic fraction of DE soot is mutagenic for bacteria and mammalian cells and is carcinogenic for mouse skin. The role of soot-associated mutagenic organic compounds in the carcinogenicity of inhaled DE in rats is an issue in interpreting bioassay results and extrapolating health risks to man. This study tested the importance of soot-associated mutagenic organic compounds in the carcinogenic response of rats exposed chronically by inhalation to high concentrations of DE by comparing the pulmonary carcinogenicities of DE and organic mutagen-poor carbon black (CB). F344 rats were exposed 16 h/day, 5 days/wk for 24 mo to DE or CB at 2.5 or 6.5 mg particles/m^3, or to clean air as controls. The DE soot was 8 % extractable (by mass) with dichloromethane, while the CB was 0.12 % extractable. DE soot extract contained three orders of magnitude more Ames mutagenic activity per gram than CB extract. Particle clearance overloading, macrophage hyperplasia, epithelial proliferation, inflammation, and fibrosis occurred in all groups exposed to DE or CB. Lung burdens of particles reached 21 and 39 mg for low and high CB exposures, and 41 and 85 mg for low and high DE exposures, respectively at 23 mo. The combined prevalences of benign and malignant neoplasms in rats dying or sacrificed after the end of exposures were 18 and 11 % for the low DE and CB groups, and 57 and 67 % for the high DE and CB groups, respectively. These similar prevalences suggest that DE and CB were equally carcinogenic, using exposure concentration as the dose term. Using lung burden as the dose term, CB was more carcinogenic than DE. These results suggest that soot-associated, mutagenic organic compounds are not the major factor in the carcinogenic response of rats to chronic, high-level exposure to DE.

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A 42

Are Mixed Coal Mine Dusts Inert or Toxic; New Results to a Classical Unresolved Experimental Question

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Epidemiological investigation could clearly show that workers in hard coal mining have a significant different risk in contracting pneumoconiosis. The risk is higher in places where dusts with low quartz content were present (<1.5%) in comparison to other places with higher quartz percentages (>10%). Animal testing of those dusts didn't show corresponding grading when the samples were injected intratracheally or intraperitoneally; in particular the coalrich dust exerted no toxic effects even after a 50 mg/animal dose intratracheally. The silica always disclosed as the main toxic principle in these mixed dusts.

Testing two samples of authentic dusts with contrasting minerai/quartz content (A: <1% vs. B: 12% silica) in short time inhalation of 2 x 5 days showed 90 days after finishing inhalation significant increases of collagen fibres in the lung parenchyma also in the low quartz sample A. In addition the lung responses to both samples in further parameters such as cells in the BAL, lung dust clearance and amount and composition of surfactant were qualitatively different.For the first time experimental evidence is given for the harmfulness of coal dust low in quartz. We conclude that only the inhalation model is capable to produce a response to this type of dust which is in agreement to human epidemiological knowledge.

A 43

Influence of the Nature and the Dose of Dusts, on Lung Clearance. Experiences with a Short Time Inhalation Model

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The Essen short time inhalation model comprises a 2 x 5 day/5 hr inhalation of dust and postexposure observation time up to 90 days. Using rats or mice short time effects as well as the beginning of chronification can be evaluated using parameters from the brochoalveolar lavage, morphological changes measured by quantitative morphometric procedures and dust retention.

In the following data on the elimination testing various kinds of dusts and different dust doses are shown. We tested model dust such as silica, clay minerals, silicon carbide and authentic mixed dust from working places. For comparative evaluation of these dusts the similarity of the grain size distribution of the tested samples is a basic requirement. This can be achieved by specially developed preparation techniques. A striking result of all inhalation studies was the strong influence of the dust nature on the lung clearance. This was also effective with high concentrations, which are generally attributed to an overload situation.

We conclude that this model is highly efficient for the investigation of lung clearance. In our opinion studies on the kinetics of dust elimination is a sine qua non for correct toxicological dust evaluation.

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A 44

Mechanism of the Induction of Fibrosis

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Acute injury to the lung usually heals without consequence. Continuing injury, eg inhalation of toxic particles, may lead to fibrosis, the outcome depending upon toxicity, dose and the effectiveness of lung defense in limiting cell-particle interactions. The predominant interstitial location of fibrosis raises a question: Does macrophagic secretion of fibrogenic factors occur within the alveolus or in the interstitium? Experimental evidence favors the interstitial location. Failure of alveolar macrophages to clear inhaled particles increases the probability of trans-epithelial passage to the interstitium where the particles interact with macrophages initiating fibrosis. Transport across the epithelium is bidirectional; increased egress of macrophages or PMN in pneumonia reduces the interstitial particulate load; similarly, an experimentally controlled inflammatory response clears interstitial particles and reduces the fibrotic response.

2057355132

A 45

Sequence of Events in Carcinogenesis - Multistage Carcinogenesis, Initiation, Promotion, Progression, Protooncogenes and Tumor Suppressor Genes

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The development pattern of most cancers is best described by a multistep mechanism involving 5 to 6 independent alterations. The predominant cause of the first of these steps is a genotoxic agent, often referred to as an initiator. Subsequent (promotion and progression) events arise due to additional genotoxic and epigenetic alterations. As a result, the cells acquire numerous gene dysfunctions. Recent advances in carcinogenesis have provided the framework for understanding how these gene aberrations result in oncogenic transformation. Specifically, the majority of the dysfunctional genes are aberrant forms of genes that control cell proliferation. Their normal forms are referred to as protooncogenes and tumor suppressor genes. The former are dominant-acting genes that code for substances that enable the cell to respond to division stimulants. Dysfunction of a protooncogene, thus, results in the cell being continually induced to divide. On the other hand, tumor suppressor genes provide the cell with proteins that govern the rate of cell division. Consequently, dysfunction of these genes results in cells with impaired ability to regulate division processes. Recently, there has been considerable advancement in eliciting the temporal sequence of genetic alterations in colon cancer. Acquisition of this information for respiratory cancers is currently the goal of several laboratories. Knowledge of the gene dysfunctions that are common in the early stages of disease would facilitate the development and use of new intervention protocols, well in advance of clinical cancer. This presentation will review the gene dysfunctions documented for respiratory cancers and describe recent investigations focused on delineating the temporal evolution of these gene alterations. (Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under contract No. DE-AC04-76EV01013.)

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A 46

Influences of Gender, Strain, and Species Differences in Pulmonary Toxicological Assessments of Inhaled Particles and/or Fibers

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Two significant challenges for inhalation toxicologists are 1) the study of mechanisms of particulate-induced lung disease and 2) the extrapolation of experimental animal data to humans for risk assessment determinations. These estimates are generally dependent upon data for which a variety of species and strains are used. Numerous examples from the literature demonstrate significant sex, strain, and species differences in pulmonary responses following inhalation of identical materials. Accordingly, some of these differences reported for selected materials are summarized herein. Rats and hamsters have been used for a variety of chronic inhalation studies including asbestos and refractory ceramic fibers. Fiber-exposed hamsters are sensitive to the development of mesotheliomas but have a relative absence of lung tumors. The converse, with some exceptions (e.g., erionite), generally is true for fiber-exposed rats. In another example, chronic inhalation studies have been carried out with diesel exhaust, carbon black or talc particles, using rats and mice. In each case, an increase in lung tumors was observed in rats, while similarly exposed mice appeared to be relatively unresponsive. In addition, short-term inhalation experiments with rats or mice exposed to crystalline silica particles demonstrated that pulmonary inflammatory responses in rats were sustained and progressive while inflammation in the lungs of mice was minor and transient. In a study comparing the pulmonary responses to silica in Fischer 344 and CD rats, it was shown that CD rats developed greater inflammatory responses as measured by pulmonary lavage, while histopathologic effects in Fischer rats were more vigorous. These results point out the complexity of pathogenic pulmonary processes in response to silica inhalation. Finally, chronic inhalation studies with Kevlar® fibrils, titanium dioxide, chromium dioxide, or titanium tetrachloride particles in CD rats have clearly demonstrated an increased incidence of proliferative keratin cysts in females relative to males. None of the disparities outlined above can be accounted for solely by dose or by differences in alveolar macrophage clearance functions. Therefore, until more information is forthcoming regarding sex, strain, or species differences in lung responses to inhaled particulates, it will be prudent to consider the most sensitive factors for extrapolation to humans.

2057358133

A 47

The Effect of Chemical Composition on the Solubility of Glass Fibres In Vivo and In Vitro

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The dissolution rates of a range of experimental and production glass fibres of varying diameters and chemical compositions were measured in vitro. Five glass fibre samples were subsequently selected for in vivo studies to assess the fibres durability within the mammalian lung. The composition of these fibre samples covered the extreme range of dissolution rates measured in vitro.

Seventy mg samples of each fibre type were exposed within a nuclear reactor to activate the sodium component. Suspensions containing 1.2 mg of the activated fibres were instilled via the trachea into the lungs of Fischer-344 rats. At 2 d post-instillation (PI), the mass and number of fibres within the lung was estimated by thoracic gamma scintillation counting of the activated ^{24}Na component.

Groups of animals were killed at various times from 2 to 360 d PI. The lung of each animal was excised and digested in 5 % sodium hypochlorite solution, for 4 h at 4° C. Aliquots of lung digest were filtered, and the filters cleared for fibre size and number characterisation using optical phase contrast microscopy (OPCM) and semi-automatic image analysis. In addition, morphometry and composition was assessed by electron probe microanalysis (EPM) using a scanning electron microscope (SEM).

Both the numbers and dimensions of the fibers which were most durable in vitro were unchanged in vivo up to 360 d PI. Morphometric changes in the least durable fibres were apparent after only 14 d exposure within the lung, and the effect was most noticeable in the longer fibres (>15 µm). It appeared that the shorter fibres (<10 µm) were protected from dissolution by being fully engulfed within macrophages. This suggests that the more acidic intra-macrophage environment reduced the fibre dissolution rate. After one year, only 10 % of the least durable fibres remained within the lung.

The in vivo data suggests that the fibre types found to be relatively soluble in vitro have quite short residence times in mammalian lung.

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Long-Term Clearance of Ceramic Fibers from Guinea Pig Lungs

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The fibrogenic and carcinogenic effects of mineral fibers have been shown to be related to their durability as well as their dimensions. Therefore, evaluation of the factors influencing pulmonary deposition and clearance of ceramic fibers is essential for testing the safety of this product. Evaluation of pulmonary deposition and clearance of ceramic fibers in the lungs of Guinea Pigs (GP) will be presented in this report.

Male Hartly albino GP were exposed to ceramic fibers for six hours per day for 5 days. Animals were sacrificed at 5, 45, 90 and 180 days after exposure. Lung lobes were removed and ashed in a low temperature asher. The ash was washed and filtered on membrane filters. Fiber counting and sizing in both air and biological material were performed using phase contrast microscopy, with a 10 x eyepiece (fitted with a Porton graticule) and 100 x objective (1.3 N.A.). That is 1000 x total magnification.

Average concentrations of airborne fibers during exposure was 225 f/cc with a count median length of 13.4 µm and geometric standard deviation of 2.5. The count median diameter was 0.9 µm with a geometric standard deviation of 1.9. The total number of fibers recovered from the lungs at 5, 45, 90 and 180 days were 19.8 ± 2.3, 12.8 ± 1.3, 9.1 ± 1.0 and 3.8 ± 0.88 million fibers per GP, respectively. These results indicate that fibers retained after six months are about 20 percent of the original lung burden.

2057355134

Biopersistence of Crocidolite vs. Manmade Vitreous Fibers (MMVF) in Rat Lungs After Brief Exposures

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Biopersistence of commercial MMVF and crocidolite fibers were studied in male Fischer 344 rats. MMVF used were size-selected to be rat-respirable, and rats were exposed nose-only 6 hours/day for 5 days to two different fiber glass compositions, a rock wool (basalt-based), a slag wool, to crocidolite, or to filtered air. Sacrifices occurred at 1 hour, 1, 5, 31, 90, 180, 270, 365 and 545 days after exposure stopped. Fibers were recovered from digested lung tissue to determine changes in concentrations (fibers/dry lobe) and fiber retentions (expressed as percent of day 1 retention, PR) for selected dimensions. One day average concentrations of retained MMVF and crocidolite fibers 0.5 µm or thicker, or of fibers 10 µm or longer, or of those longer than 20 µm were roughly equal, permitting direct comparisons between MMVF and crocidolite. At 270 days average PR for fibers 0.5 µm or thicker were from 5 to 9 ± 3 % for MMVF and 39 ± 12 % for crocidolite; for fibers 10 µm or ≥, PR were 1 to 15 ± 10 % for MMVF and 32 ± 4 % for crocidolite. At 180 days PR for MMVF ≥ 20 µm ranged from not detected to 9 % and were at background levels before or by 545 days. PR for crocidolite fibers ≥ 20 µm generally exceeded 50 % from 90 days through 545 days. Among the MMVF, PR for long rock wool fibers were elevated through the first 90 days compared to the PR for long glass and slag wool fibers. At 31 and 90 days, for rock wool fibers ≥ 20 µm, PR were 113 ± 27 % and 45 ± 18 %, respectively, while PR for the other MMVF ranged from averages of 10 to 23 ± 18 % (31 days) and from 1 to 9 ± 14 % (90 days). These results suggest strongly that MMVF dissolved or broke in vivo whereas crocidolite fibers did not change.

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Chronic Inhalation Toxicity of Fibrous Glass in Rats

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This study was initiated to determine the chronic biological effects in Fischer 344 rats of size-separated respirable fractions of Fibrous Glass (FG) having compositions representative of common building insulation wools. Rats were exposed using nose-only inhalation chambers, 6 hrs/day, 5 days/week, for 24 months to three concentrations (3, 16, and 30 mg/m^3) of two different compositions of FG, or to filtered air (negative control). Positive control animals were exposed to 10 mg/m^3 of chrysotile asbestos. FGs were size selected to be respirable in the rat and the aerosol generation technique did not alter the dimensions of the fibers. Interim sacrifices took place at 3 to 6-month intervals to monitor progression of pulmonary changes. Fibers were recovered from digested lung tissue for determination of changes in fiber number and morphology. In animals exposed to the highest concentration of one composition of FG (MMVF 11), $6.4 \pm 3.1 \times 10^5$ fibers/mg dry lung tissue were recovered after 24 months of exposure. Exposure to chrysotile asbestos (10 mg/m^3) resulted in pulmonary fibrosis as well as significant increases in lung tumors and a mesothelioma. FG exposure was associated with a non-specific inflammatory response in the lungs that did not appear to progress after 6 - 12 months of exposure. These cellular changes are reversible and are similar to the effects observed after inhalation of an inert dust. No lung fibrosis was observed in the FG exposed animals. Further, FG exposure resulted in no mesotheliomas and no statistically significant increase in the lung tumor incidence when compared to the negative control group. Modeling of the lung burden data provided information on the clearance of FG from the lung after exposure was stopped.

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Chronic Inhalation, Intratracheal and Biopersistence Studies on Refractory Ceramic Fibers (RCF)

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RCF's are man-made vitreous fibers used as insulation primarily in high temperature industrial applications. Lifetime nose-only inhalation studies and a lifetime intratracheal study were conducted on Kaolin RCF to assess potential toxic effects. Fischer 344 rats were exposed 6 hours/day, 5 days/week for 24 months to concentrations of 3, 9, 16 and 30 mg/m^3 while Syrian hamsters were exposed to 30 mg/m^3 for 18 months. Intratracheal administration of 2 mg of size-selected RCF in 0.2 ml of saline was also conducted in F344 rats. Numerous interim sacrifices and recovery groups allowed assessment of the progression of pathological lesions, lung burden clearance and retention. In addition to pulmonary fibrosis, Kaolin RCF by inhalation produced a 42 % incidence of mesothelioma in hamsters, but no lung tumors. In rats, excess lung tumors (13 %) occurred at 30 mg/m^3 but not at lower doses. Two (1.6 %) mesotheliomas occurred at 30 mg/m^3 and one (0.8 %) at 9 mg/m^3. Intratracheal administration produced granulomas, fibrosis but no excess in lung tumors and no mesotheliomas. Modelling of the inhalation data shows a half-life of approximately 200 days and suggests clearance at 30 mg/m^3 may be inhibited compared to that at lower doses.

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A Comparison of the Effects of Chrysotile and Crocidolite Asbestos in Rats after Inhalation

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Male F344 rats were exposed via nose only inhalation to 10 mg/m^3 chrysotile (CHRY) or crocidolite (CROC) asbestos for 6 hr/da, 5 da/wk: CHRY - 24 mo; CROC - 10 mo followed by no exposure until ~20 % survival. The number of respirable CHRY fibers (< 3.0 µm diameter x > 5.0 µm length) was ~10 X greater than CROC: CHRY = 1.2 X 104/cc; CROC = 1.2 X 10^3. Groups of 3 or 6 rats were removed at 3, 6 and 9, 12, 18 and 24 mo for lung histopathology. Decreased body weight gain and increased mortality were observed with CROC up to 10 mo after which it was comparable to CHRY. Both types of fibers caused progressive pulmonary lesions, i. e. macrophage infiltration, bronchiolization of proximal alveoli and interstitial fibrosis. However, the lesions occurred earlier and were more intense with CROC than CHRY. Pleural fibrosis was observed by 10 mo after CROC but not CHRY exposure, even after 24 mo. Both types of asbestos fibers were clearly carcinogenic for the rat lung causing an increased incidence of broncho-alveolar adenomas and carcinomas. The number of fibers/mg dry lung after ashing was greater for CHRY than CROC. The results of this study show that CROC is considerably more pathogenic to lungs of rats than CHRY asbestos even when the exposure is shorter.

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Biopersistence and Pulmonary Effects of Inhaled Kevlar® or Wollastonite Fibers Following Short-Term Exposures

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Fiber dimension and durability are important features in the development of pulmonary carcinogenic and fibrogenic effects. Using a short-term inhalation bioassay, we have studied pulmonary clearance patterns and evaluated the pulmonary toxicity of wollastonite fibers and Kevlar® para-aramid fibrils relative to other reference materials. Rats were exposed for 5 days to aerosols of Kevlar® fibrils (900 - 1344 f/cc; 9 - 11 mg/m^3) or wollastonite fibers (800 f/cc; 115 mg/m^3). Following exposures, cells and fluids from groups of sham- and fiber-exposed rats were recovered by pulmonary lavage or lungs were infused for morphological assessments. The lungs of additional exposed rats were digested to quantify dose, fiber dimensional changes over time and clearance kinetics. Five-day exposures to Kevlar® fibrils or to wollastonite fibers elicited a transient neutrophilic inflammatory response with concomitant increases in BAL fluid levels of alkaline phosphatase, NAG, LDH, and protein. Unlike the data from silica and asbestos exposures wherein inflammation persisted, biochemical parameters returned to control levels between 1 week and 1 month postexposure. The results from digested lung tissue showed that inhaled wollastonite fibers were cleared rapidly with a clearance half-time of approximately 1 - 2 weeks. Mean fiber lengths decreased from 11 µm to 6 µm over a 1-month period. Mean fiber diameters increased from 0.5 µm to 1.0 µm over this time period. Fiber clearance studies with Kevlar® demonstrated a transient increase in the numbers of retained Kevlar® fibrils at 1 week postexposure, with rapid clearance of fibers thereafter. A progressive decrease in the mean lengths and diameters of inhaled Kevlar® fibrils was measured over a 6-month postexposure period indicating shortening of the fibers. Mean fiber lengths decreased from 12.5 µm to 7.5 µm; mean fiber diameters decreased from 0.33 µm to 0.24 µm. The percentages of fibers greater than 15 µm in length decreased from 30 % immediately after exposure to 5 % at the 6-month postexposure period; the percentages of fibers in the 4 - 7 µm range increased from 25 % immediately after exposure to 55 % after 6 months. These data suggest that inhaled Kevlar® or wollastonite fibers are degraded in the lungs of exposed rats, and this may be responsible for the differences in toxicity observed between these fiber-types and durable dusts such as silica or crocidolite asbestos fibers.

Source: https://www.industrydocuments.ucsf.edu/docs/gpgl0000

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Squamous Lung Lesions Associated with the Chronic Exposure by Inhalation of Rats to p-Aramid Fibrils and to Titanium Dioxide: Findings of a Pathology Workshop

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The last few years have seen discussions surrounding the morphologic diagnosis, prognosis and interpretation of risk evaluation to humans of squamous lesions occurring in the lungs of rats following chronic inhalational exposure. In an effort to help clarify this situation for two substances, p-aramid fibrils and titanium dioxide, an international panel of pathologists with experience in both veterinary and human lung pathology met to evaluate these lesions. Prior to the meeting, each participant had been sent a series of slides representing the entire spectrum of squamous-like lesions encountered in investigations with these two substances. The specific questions each participant was asked to address related to the morphological classification of the lesion and its possible relevance to humans.

There was unanimous agreement that the lesions, from both series of studies, at their most marked, could best be described as proliferative keratinous cysts. Whilst all participants agreed that the lesion was not a malignant neoplasm, and the majority felt that the lesion was not even neoplastic, a minority considered the lesion was probably a benign tumour.

A detailed histopathological presentation of the material and reasoning behind this overall evaluation will be illustrated in the presentation.

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Toxic and Chromosome Damaging Effects of Natural and Man-Made Mineral Fibers in Epithelial Lung Cells In Vitro

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Much is known about the correlation between asbestos exposure, especially under working conditions, and the resulting health damage in the respiratory tract. But there is less information about the influence of artificial fibers such as from glass and ceramics. Seven fibrous dusts were investigated with regard to their toxicity and their ability to induce chromosomal damages. The cell system used for this investigation was a fetal epithelial lung cell line derived from the Syrian golden hamster. Cytotoxicity, measured by evaluating the mitotic index, was strongest in cultures treated with the man-made fibrous dusts silicon carbide and JM 104/475, B1M and B3K glass fibers, whereas the naturally occurring chrysotile and crocidolite asbestos fibers and erionite fibers showed a considerably lower toxic potential.

The occurrence of chromosomal lesions was measured by counting micronuclei. All the tested dusts induced dose-dependently enhanced micronucleus frequencies, with chrysotile, silicon carbide and JM 104/475 glass fibers being the most effective. Special analysis of the produced micronuclei yielded information about their origin. B1M and B3K glass fibers, erionite and silicon carbide were shown to induce a majority of the micronuclei via an aneugenic activity, i.e. by chromosome malseparation, whereas the asbestos fibers chrysotile and crocidolite as well as glass fibers JM 104/475 exhibited an clastogenic rather than aneugenic activity.

The results indicate the ability of different fibrous dusts to influence the chromosomal integrity of living cells in consideration of the portion of critical fibers in the samples. The mechanism by which those different particle dusts induce abnormal chromosome sets are not necessarily identical. The relevance of the observed genetic lesions with respect to carcinogenicity is well known, thus the observed differential effects should at least be considered when the carcinogenic potential of dusts is discussed.

Source: https://www.industrydocuments.ucsf.edu/docs/gpgl0000

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Cellular Effects of Mineral Fibres

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Asbestos and other mineral fibres including glass fibres have been shown to cause tumours in experimental animals and man. The toxic and carcinogenic potency of the fibres has been related to their physical and chemical properties. The mechanisms responsible for the biological effects are still not known in detail. Therefore three mineral fibres have been investigated for different biological effects in hamster airway epithelial cells to elucidate interactions between possible target cells and fibres.

Crocidolite asbestos and two glass fibres JM 104/475 and B-1M were examined in this study. Crocidolite fibres and the glass JM 104/475 fibres have a similar fibre size distribution, whereas glass B-1M fibres are generally larger in size. The different fibre samples have been tested for their toxic, genotoxic and carcinogenic activity. As further biological parameters, certain components of the cytoskeleton were examined for reversible or irreversible lesions induced by fibrous dusts. The cytoskeleton is of major importance for the structural integrity and physiologic processes in the cells. In the epithelial cell system investigated, both crocidolite asbestos and the glass fibre JM 104/475 induced a dose-dependent decrease in cell survival and colony-forming efficiency whereas the glass fibre B-1M revealed only weak toxic effects. Transformation to anchorage independent growth could only be seen after treatment of the cells with crocidolite asbestos. All fibre samples induced chromosomal damages as shown by increased frequencies of micronuclei formation. The application of crocidolite asbestos was followed by changes in the actin and vimentin systems of the cells, whereas the actin and microtubule organisation were affected by the glass fibres.

The study shows that the biological alterations after application of fibres can not only be traced back to size and chemical composition of the fibres tested or on induced genotoxic effects. Other factors, e.g. disturbances of basic physiologic cellular functions via damage to at least parts of the cytoskeleton, might considerably contribute to the toxic effects of different fibres.

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Increased Incidence of DNA Double-Strand Breaks and Anti-DS DNA Antibodies in Blood of Workers Occupationally Exposed to Asbestos Fibers

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Asbestos workers have an increased risk of lung cancer, mesothelioma, and other tumours. Although it used to be believed that mesothelioma spreads mainly by direct extension, hematogenous metastases are frequently found at autopsy. Hematogenous metastases to the liver, bone, adrenal gland, kidney or brain were observed. This supports the view that mesothelioma is a systemic disease, and that this should be taken into account. Asbestos, proven to be carcinogenic in humans and animals, is reported to have no genotoxic effect. Earlier findings showed that crocidolite can induce DNA strand breaks in cultured cells.

We investigated DNA double-strand breaks and anti-ds DNA (double-stranded DNA) antibodies in blood of 16 workers occupationally exposed to asbestos. According to our results obtained with neutral filter elution, individuals who had been exposed to asbestos fibers showed twice to four times more DNA double-strand breaks in white blood cells than 30 non-exposed persons. The induced DNA fragments are of about 250 kb (compared to chromosomal DNA of Saccharomyces cerevisiae standard marker). Using additionally the chromosomal DNA protective method of agarose-plugs, DNA fragments in the range of 200 to 1000 kb have been found in the white blood cells of the same ten workers occupationally exposed to asbestos. In the white blood cells of non-exposed subjects no DNA fragments could be detected with this method. Compared to 51 non-exposed persons, elevated anti-ds DNA antibody concentrations were found in the same ten workers occupationally exposed to asbestos.

The fact that workers occupationally exposed to asbestos have distinctly more double-strand breaks and anti-ds DNA antibodies could mean that an increased incidence of DNA-fragments may be an important indicator in the systemic effect of asbestos-associated carcinogenesis. Apparently, the chronic effects of asbestos observed here do not seem to be indentical with that of previously reported acute in vitro effects.

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